

"Novel agents for the prevention of *leishmaniasis*"

The invention relates to novel agents for the prevention of *leishmaniasis* in animals and in humans.

5

It relates in particular to nucleic acid molecules encoding virulence or pathogenicity factors in *Leishmania* and to the use thereof for producing such factors in order to develop vaccine compositions
10 against *leishmaniasis*.

Leishmaniasis represents one of the six major parasitic diseases and is considered, in this respect, to be a priority by the World Health Organization (WHO).
15 *Leishmania* exists in the extracellular promastigote form, inside the digestive tube of the vector insect (the sandfly), and in the intracellular amastigote form, in the mammalian host. Several molecules, including lypophosphoglycans (LPGs) or a metallo-
20 protease called gp63, appear to play an important role in the infectious capacity and the the pathogenicity of the parasite. More recently, a family of glycoproteins, called promastigote surface antigens (PSAs), has raised new interest. These PSAs are characterized by the
25 presence of leucine-rich repeats that can be involved in protein/protein interactions and confer Th 1-type cell-mediated protective immunity in mice. In organisms, such as bacteria or plants, it appears that PSAs were involved in functions such as cell adhesion,
30 resistance to pathogens and signal transduction.

However, no biological role has been described or suggested in *Leishmania*.

35 It has been possible for this role to be studied by the inventors by means of the technique in their possession for culturing *Leishmania* promastigotes and amastigotes under serum-free and axenic conditions, with a

completely defined medium, i.e. in which the constituents are all defined, and which is the subject of patent FR 93 05 779 of May 13, 1993, in the name of IRD (ex ORSTOM). The mastering of this method allows
5 them to have parasitic forms free of the contaminants introduced up until now by the culture media, and antigenic determinants in a highly purified form.

In said applicant's FR patent, the isolation and the
10 identification of an excreted/secreted PSA (excretion/secretion antigen, abbreviated to ESA) of 38 kDa and of 45 kDa in the culture supernatant of *L. amazonensis* have already been described.

15 The inventors have presently isolated and cloned the cDNA encoding this protein and evaluated its role in the biology of the parasite by developing an additional transgenesis strategy. These studies have made it possible to demonstrate the involvement of this PSA as
20 a virulence and/or pathogenicity factor and to develop constructs for overexpressing the *Leishmania* gene encoding this PSA, which makes it possible to develop agents for producing vaccine compositions against *leishmaniasis*.

25 The aim of the invention is therefore to provide nucleic acid sequences being capable of encoding PSAs of promastigote forms and of amastigote forms of *Leishmania*, constituting virulence and/or pathogenicity
30 factors.

It is directed more particularly toward providing vectors for the overexpression of these PSAs, and also genetically modified parasites.

35 The invention is also directed toward the culture medium supernatants of the PSAs obtained, and also the isolated, purified PSAs, and the beneficial use of their properties for developing vaccine compositions

against *leishmaniasis*.

The nucleic acid sequences of the invention correspond to isolated nucleic acids capable of encoding a PSA of
5 promastigote forms or of amastigote forms of *Leishmania*, said nucleic acids corresponding to one of the sequences SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4, SEQ ID No 5 and SEQ ID No 11, and encoding PSAs having the sequences, respectively, SEQ ID No 6,
10 SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10 and SEQ ID No 12.

The nucleic acid sequences of the invention are more especially sequences of cDNA clones belonging to a
15 family corresponding to the characteristics illustrated by figure 2 and comprising in particular a *SalI* restriction site and two *HindIII* restriction sites, with a stop codon located downstream of the first *HindIII* site.

20 The invention is directed in particular toward the cDNA clones of said family comprising an *EcoRV* and/or *PstI* restriction site between the two sites *SalI* and *HindIII*, or on either side of the *SalI* site.

25 The invention is also directed toward the isolated immunogenic proteins, characterized in that they have a sequence as encoded by the nucleic acids defined above. It is directed in particular toward the proteins
30 corresponding to the sequences SEQ ID No 6, SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10 or SEQ ID No 12.

35 These proteins belong to the "promastigote surface antigen" (abbreviated to PSA) family and possess characteristic regions illustrated in figures 3A and 3B. These proteins can be post-translationally modified by means of N-glycosylations, phosphorylations and anchoring of a GPI. They possess a hydrophobic signal

peptide in the carboxy-terminal position.

The inventors have obtained constructs that make it possible to express the sequences defined above, in the sense position, in an expression vector, by directional cloning of said sequences.

The invention is therefore directed toward nucleic acid constructs, characterized in that they comprise isolated nucleic acids in the sense position, capable of encoding an immunogenic protein of promastigote forms or of amastigote forms of *Leishmania*, these proteins corresponding to one of the sequences SEQ ID No 6, SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10 and SEQ ID No 12.

The invention is directed in particular toward the nucleic acid constructs comprising sequences of cDNA clones belonging to a family corresponding to the characteristics illustrated in figure 2 and comprising in particular a *SalI* restriction site and two *HindIII* restriction sites, with a stop codon located downstream of the first *HindIII* site.

The cDNA clones comprising an *EcoRV* and/or *PstI* restriction site between the two sites *SalI* and *HindIII*, or on either side of the *SalI* site, are particularly preferred.

Particularly advantageous constructs comprise, as nucleic acid sequences, a sequence chosen from SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4, SEQ ID No 5 and SEQ ID No 11, these sequences encoding, respectively, proteins having the sequences SEQ ID No 6, SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10 and SEQ ID No 12.

Preferred constructs comprise said nucleic acid sequences in a rapid multiplication plasmid such as

pTex.

The invention is also directed toward the *Leishmania* strains transfected with such constructs, whether
5 promastigote forms or amastigote forms are involved.

Transfected strains that are preferred, taking into account the vaccine applications targeted, are
10 *L. infantum* strains.

Advantageously, the PSAs are produced in large amount, constitutively, in the parasites.

The invention is also directed toward a method of
15 transfecting a *Leishmania* parasite, characterized in that a vector as defined above, comprising a marker, is introduced into the *Leishmania* parasite, the transfected parasites are selected by means of said marker, they are placed in culture in a completely
20 defined axenic and serum-free medium, and the culture supernatant which contains the immunogenic proteins present in concentrations of the order of 10 to 20 times higher than that produced by the *Leishmania* mother strain is recovered.

25 The introduction of the vector into the parasite is, for example, carried out by electroporation.

The insertion of these nucleic acids into the parasites
30 makes it possible to increase the infectious capacity of the latter: their ability to survive in the infected macrophage and to multiply therein is up to 5 times greater than that of the parasite not transfected with such nucleic acids.

35 Said PSAs are produced in large amount in the parasite culture medium supernatant. The invention is therefore also directed toward the culture medium supernatants of said genetically modified parasites, and also the PSAs

isolated from these supernatants and purified.

The invention thus provides agents of great value for satisfying the industrial demand for large amounts of proteins constituting virulence/pathogenicity factors in *Leishmania*.

Due to their immunogenic capacity, these proteins make it possible to obtain, after immunization of animals according to conventional techniques, polyclonal antibodies and to develop monoclonal antibodies. The immunization of mice has thus made it possible to obtain anti IgG2A antibodies and that of dogs has made it possible to obtain IgG2 antibodies.

The invention is therefore also directed toward such antibodies and makes beneficial use of their properties for developing, on an industrial exportable scale, vaccine compositions against *leishmania* in humans or animals.

The diagnostic applications of these antibodies are also part of the invention.

Other characteristics and advantages of the invention will be given in the examples which follow, in which reference will be made to figures 1 to 8, which represent, respectively:

- figure 1, the 3' alignment of the nucleotide sequences of cDNA clones according to the invention;
- figure 2, a recapitulative diagram of the nucleotide sequences of the cDNA clones obtained after immunoscreening, with an anti-ESA monoclonal antibody, of *L. amazonensis* promastigote form and amastigote form expression libraries. The restriction enzyme sites are indicated above each sequence;
- figure 3A, the location of various protein regions, characterized by their specific amino acid

composition, present on the protein sequence deduced from the cDNA of the clone A3B; and figure 3B, a diagrammatic representation of the protein sequence deduced from the cDNA of the clone A3B encoding a PSA;

- 5 - figure 4, the analyses of the transcripts by RT-PCR in the promastigote (P) and amastigote (A) forms;
- figure 5, the level of production of the protein by Western blotting, using an anti-PSA antibody;
- figure 6, the effect of the overexpression of a
10 PSA of *L. amazonensis* on the infectious capacity of the parasites;
- figure 7, the nucleotide sequences SEQ ID Nos 1 to 5 and 11, respectively, of the clones A3B, 2C1, 1A1, 2G1 and W2 of *L. amazonensis* promastigotes and
15 amastigotes and IJ11 of *L. infantum* promastigotes, and the corresponding encoded amino acid sequences SEQ ID Nos 6 to 10 and 12, and
- figure 8, the parasitic index determined during the *in vitro* infection of canine macrophages with a
20 wild-type strain or selected *L. infantum* promastigote clones, at various incubation times.

1 - Molecular characterization of the major immunogens of the ESAs of promastigote and amastigote forms of
25 *L. amazonensis* (abbreviated to *Lma*)

This characterization was carried out by screening *L. amazonensis* promastigote form and amastigote form cDNA expression libraries using a monoclonal antibody directed against the ESA major immunogen.

30 - cDNA library characteristics:

Two cDNA expression libraries, respectively of promastigote forms and of amastigote forms of *L. amazonensis*, were produced. The characteristics of
35 these libraries are given in table I. The exponential-phase and stationary-phase parasites were mixed in order to have access to the various transcripts that may be expressed during the various stages of the *in vitro* culturing thereof. 5×10^4 phages per library were

then immunoscreened with the monoclonal antibody F5 diluted to 1/500. The production of this antibody is the subject of the example in the FR patent mentioned above.

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Table I

cDNA library Lma LES D4 + D7	Promastigotes	Amastigotes
Harvest D4 + D7	$7.8 \cdot 10^9$	$7.8 \cdot 10^9$
Titration after packaging	350 000	500 000
Titer after amplification	$8.32 \cdot 10^7$ pH/ul	$2.16 \cdot 10^8$ pH/ul

D4 + D7 = parasites harvested on the 4th day, in the exponential phase, and on the 7th day, in the stationary phase of their growth.

10

- Isolation and sequencing of the clones recognized by the monoclonal antibody F5

13 clones of the promastigote library were found to be positive and 11 clones of the amastigote library were found to be positive. All these clones were isolated by secondary and tertiary screening.

The plasmid DNA of all the clones isolated was analyzed after various enzymatic digestions and the cDNAs having larger inserts, by *EcoRI/XhoI* digestion, were selected in order to eliminate the cDNAs that were too truncated in the 5' position. As shown in table II, the clones 1A1, 1B1, 2B3, 2C1, 2D1 and 2E1 of the promastigote cDNA library and the clones A3B, V4A, V5, W2 and W3 of the amastigote library exhibit the larger inserts.

The analysis of these clones, by determining the presence or absence of two previously selected restriction enzyme sites (*HindIII* and *SalI*), show that they exhibit strong homology of their nucleotide sequence.

Three different classes of clones were demonstrated, by double *HindIII/SalI* digestion, with a *HindIII/SalI*

fragment less than 400 bp in size (clone 2G1), 500 bp in size (clones of type 2C1 and A3B) or 600 bp in size (clones of type 1A1 or W2), respectively. Thus, five types of clones, chosen according to the specific characteristics of their DNA (the size of the insert and the location of certain restriction enzyme sites) are represented in bold characters in table II.

Table II

10 *Lma* promastigote cDNA library

cDNA clones	1A1	1B1	1C1	1D5	1F1	2A2	2B3	2C1	2D1	2E1	2F1	2G1	B3A
Size of the EcoRI/XhoI inserts (kb)	2.5	2.5	2-2.2	0.5	2	2(>)	2.5	2.4	2.4	2.4	2	1.7-2	1.7
Restriction map													
<i>Sal</i> I	Y	Y	Y	N	N	N	Y	Y	Y	Y	N	Y	N
<i>Hind</i> III	1.1	1.1	1.1	/	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
<i>Hind</i> III/ <i>Sal</i> I (bp)	600	600	500	N	N	N	600	500	500	500	N	<400	N
Recombinant protein expression													
(kDa)	45	/	40	/	/	/	/	42.5	/	/	39	?	18

Lma amastigote cDNA library

cDNA clones	A3B	V1B	V2D	V3A	V4A	V5	W1A	W1C	W2	W3	W5
EcoRI/XhoI (kb)	2.3	2-2.2	2.2	?	2.3	2.3	2	2	2.3	2.2	1.7
Restriction map											
<i>Sal</i> I	Y	Y	Y	N	Y	Y	N	N	Y	Y	N
<i>Hind</i> III	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
<i>Hind</i> III/ <i>Sal</i> I (bp)	505	500	500	N	500	500	N	N	600	500<	N
Recombinant protein expression											
(kDa)	42.5	/	36.5	/	36.5	43	/	/	45	/	/

Y = yes, for site present; N = no, for site absent;
/ = performed; ? = result not obtained.

5

Table II also gives the results relating to the ability of the clones to express a recombinant protein. IPTG was used as an inducing agent. The samples were analyzed by SDS-PAGE and immunoblotting in the presence of the antibody against promastigote form and/or amastigote form ESA, preabsorbed in the presence of *E. coli* lysate. Equivalent results are obtained. For clones of interest, the expression of various recombinant proteins ranging from 42.5 kDa in apparent molecular weight (clone 2C1), to 43 kDa (clone A3B) or 45 kDa (1A1 and W2) is noted.

The "sequence listing" document reports the results of the sequencing:

20 - of the following three types of clones of the promastigote library:

. the clone of type 1A1 (SEQ ID No 3), which expresses a protein, of sequence SEQ ID No 8, of higher molecular weight. The clones of type 1B1 and 2B3 are of the same type as this clone;

25 . the clone 2C1 (SEQ ID No 2), which expresses a

recombinant protein of molecular weight lower than that of the clone 1A1, having a sequence SEQ ID No 7;

. the clone 2G1 (SEQ ID No 4), which has the particularity of possessing a small *HindIII/SalI* fragment, which expresses a recombinant protein of molecular weight lower than that of the clone 1A1, having a sequence SEQ ID No 9;

- of the following two clones of the amastigote library:

10 . the clones of type A3B (SEQ ID No 1), which express a recombinant protein of approximately 43 kDa, of sequence SEQ ID No 6 and having a 500 bp *HindIII/SalI* fragment, the clone V5 appearing to be identical. The clones V2D and V4A are considered to be truncated clones of the same type;

. the clone W2 (SEQ ID No 5), which expresses a recombinant protein of 45 kDa, of sequence SEQ ID No 10 and which has a 600 bp *HindIII/SalI* fragment.

20 . Study of the five cDNA sequences

The alignment of the five cDNA sequences obtained is represented in figure 1, where the differences between these clones are only due to the presence of a 5'-truncated sequence and/or the insertion of sequences of approximately 72 nucleotides of the 5' side. The clones thus exhibit one (clones 2C1 and A3B) or three (clones 1A1 and W2) insertions. Outside these insertion zones, the clones exhibit homologies of the order of 99% and can be considered to belong to a cDNA family. Only the clone A3B has the ATG initiation codon, the other clones being 5'-truncated. However, the A3B cDNA does not have the 39 nt sequence encoding the "splice leader" found in the 5' position on all *Leishmania* mRNAs.

35

The cDNAs of the clones A3B and 2C1 exhibit virtually total homology and are therefore considered to be identical, the cDNA of the clone 2C1 corresponding to a 5'-truncated portion of the cDNA of the clone A3B.

The clone A3B, representative of this family, was the subject of complete sequencing in both directions.

- 5 The restriction enzyme sites for each of these clones are reported in figure 2.

The sequences SEQ ID Nos 1 to 5 correspond, respectively, to those of the cDNAs of A3B, 2C1, 1A1,
10 2G1 and W2.

- Analysis of the various deduced protein sequences

The translation of the various cDNA sequences into protein sequences was carried out by choosing the
15 reading frame corresponding to that suggested by the position of the initiation codon on the plasmid pB-SK, the transcription of which is under the control of the promoter of the *lacZ* gene subjected to induction with IPTG.

20 The A3B protein exhibits the regions illustrated in figures 3A and 3B. At the NH₂-terminal, a hydrophobic peptide, which can be cleaved, and which is described in the literature as a secretion pathway signal
25 peptide, is identified. This is followed by the leucine-rich repeat domain, the clone A3B possessing 6 repeats. About ten amino acids from the end of this domain is a region rich in proline, threonine and serine, hereinafter called poly P/T/S region. This
30 region is followed by a cysteine-rich region, that can be involved in disulfide bridges. Finally, the protein sequence ends with a hydrophobic signal peptide.

The cDNAs of the clones A3B and 2C1 exhibit virtually
35 total homology and are therefore considered to be identical, the cDNA of the clone 2C1 corresponding to a 5'-truncated portion of the cDNA of the clone A3B.

The clone A3B, representative of this family, was the

subject of complete sequencing in both directions.

The restriction enzyme sites for each of these clones are reported in figure 2: Nt = nucleotides;
5 ATG = initiation codon; TAG = stop codon.

Analysis on the PROSITE database shows that the A3B protein has an N-glycosylation site located at the end of each leucine-rich repeat domain, and 12 potential
10 phosphorylation sites.

Analysis of the location of this protein on the PSORT server predicts a cytoplasmic location at 92%, which indicates that the protein is soluble. This protein is
15 probably anchored to the surface via a glycosyl phosphatidyl inositol or GPI. The hydrophobic signal peptide can therefore be cleaved and allow anchoring of the GPI at the level of asparagine (D).

20 The theoretical molecular weight of the protein of the clone A3B differs by approximately 2.9 kDa from that of the 1A1 and W2 proteins, which is in agreement with the difference of 2.5 kDa observed between the corresponding recombinant proteins. This difference is
25 due to the presence of a variable number of leucine-rich repeats or LRRs, each also exhibiting a specific amino acid composition.

The apparent and theoretical molecular weights of the
30 four types of PSA of the invention are given in table III below.

Table III

Type of PSA	MW of the recombinant protein	Theoretical MW (nontruncated)	MW without signal peptide (3.2 kDa)
4 LRR (2G1)	/	33.5 kDa	30.3 kDa
6 LRR (A3B)	42.5 kDa	38.5 kDa	35.3 kDa
7 LRR (1A1 and W2)	45 kDa	41.4 kDa	38.2 kDa

2 - Obtaining genetically modified parasites:

Directional cloning of the LaPSA 38s gene into the expression vector pTex made it possible to obtain a construct capable of expressing the PSA gene in the sense position. The plasmid pTex-LaPSA 38s sense orientation and the empty vector pTex were then electroporated into the wild-type strain *Leishmania infantum* Mon 1 Clone 1, and the parasites were then selected with geneticin.

The study was carried out on wild-type (WT) parasites of the species *L. infantum*, those transfected with empty pTex (pTex) and those transfected with pTex containing the nucleotide sequence of interest (sense).

Molecular characterization:

The analysis of the total DNA by Southern blotting shows that the sense construct is stable and amplified in the transformed strain. The results are given in figure 4, which gives the analyses of the transcripts by RT-PCR in the two forms, promastigotes (P) and amastigotes (A). Figure 5 gives the level of production of the protein by Western blotting, using an anti-PSA antibody (figure 5A: constitutive proteins; figure 5B: excreted/secreted proteins).

Phenotypic characterization of the mutants:

The comparison of the growth kinetics between Ldi WT, Ldi pTex and Ldi Sense shows that the overexpression of LaPSA 38s does not interfere with the growth of the parasites. Only a longer lag phase is observed for the

strains transformed with the wild-type strain.

The sensitivity to lysis by human complement was also studied. Recently, it was demonstrated that *L.*
5 *amazonensis* PSA had the property of inhibiting the action of complement *in vitro*. The "sense" promastigotes are more sensitive to complement. The excess PSA at the surface of the parasites can thus lead to cleavage and also to a greater formation of
10 complexes engendering increased lysis.

Study of infectious capacity of the parasites

To study the effect of the overexpression of LaPSA 38s on the infectious capacity of the parasites, the first
15 approach consisted in bringing promastigotes of the transformed strains into contact with macrophages from dog, which is the natural domestic reservoir for visceral *leishmaniasis*.

20 Figures 6A and 6B give the results obtained, respectively, 2 h after contact with the promastigotes and 48 h after contact with the amastigotes; in these figures, the parasitic index corresponds to the % of macrophages infected with the Sense strain \times the number
25 of parasites per macrophage/% of macrophages infected with the control strain (pTex) \times the number of parasites per macrophage.

The promastigotes overexpressing LaPSA 38s exhibit
30 twice as much infectious capacity with respect to canine macrophages. Furthermore, after phagocytosis, the amastigotes expressing the transgene possess a capacity to survive and to multiply in the parasitophorous vacuole that is significantly greater
35 (2.5 to 5 times) than that of the control transfected with the empty vector.

2 - Molecular characterization of the *L. infantum*
promastigote ESAs

The nucleotide sequence of the *L. infantum* promastigote
clone IJ11 is given in figure 7 (SEQ ID No 11) along
5 with the corresponding amino acid sequence (SEQ ID
No 12).

Figure 8 reports the parasitic index determined during
the *in vitro* infection of canine macrophages with the
10 wild-type strain or the various selected *L. infantum*
promastigote form clones (MHON/MA/67/ITMAP-263, clone
2), at various incubation times. The examination of
these results shows attachment of the parasites to the
macrophages after 30 min, penetration of the parasites
15 after 2 hours and survival and multiplication of the
intracellular amastigotes at 48 hours.

CLAIMS

1. A nucleic acid construct, characterized in that it comprises an isolated nucleic acid in the sense position, capable of encoding an immunogenic protein of promastigote forms or of amastigote forms of *Leishmania*, said nucleic acid corresponding to one of the sequences SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4, SEQ ID No 5 and SEQ ID No 11.
2. The construct as claimed in claim 1, characterized in that the sequence comprises a *SalI* restriction site and two *HindIII* restriction sites, with a stop codon located downstream of the first *HindIII* site.
3. The construct as claimed in claim 2, characterized in that said nucleic acid sequence comprises an *EcoRV* and/or *PstI* restriction site between the two sites *SalI* and *HindIII*, or on either side of the *SalI* site.
4. The construct as claimed in any one of claims 1 to 3, characterized in that said nucleic acid sequence is cloned in the sense position into a plasmid such as pTex.
5. The construct as claimed in any one of claims 1 to 4, characterized in that it encodes an immunogenic protein of promastigote forms or of amastigote forms of *Leishmania*, these proteins corresponding to one of the sequences SEQ ID No 6, SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10 and SEQ ID No 12.
6. An isolated immunogenic protein, characterized in that it has a sequence chosen from SEQ ID No 6,

SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10 or SEQ ID No 12.

- 5 7. A genetically modified *Leishmania* strain, characterized in that it corresponds to *Leishmania* amastigote or promastigote forms transfected with a construct as claimed in any one of claims 1 to 5.
- 10 8. The strain as claimed in claim 7, characterized in that it is an *L. infantum* strain.
- 15 9. A method of transfecting a *Leishmania* parasite, characterized in that a construct as claimed in any one of claims 1 to 5, comprising a marker, is introduced into the *Leishmania* parasite, the transfected parasites are selected by means of said marker, they are placed in culture in a completely defined axenic and serum-free medium, and the culture supernatant which contains the immunogenic proteins present in concentrations of the order of 10 to 20 times higher than that produced by the *Leishmania* mother strain is recovered.
- 20

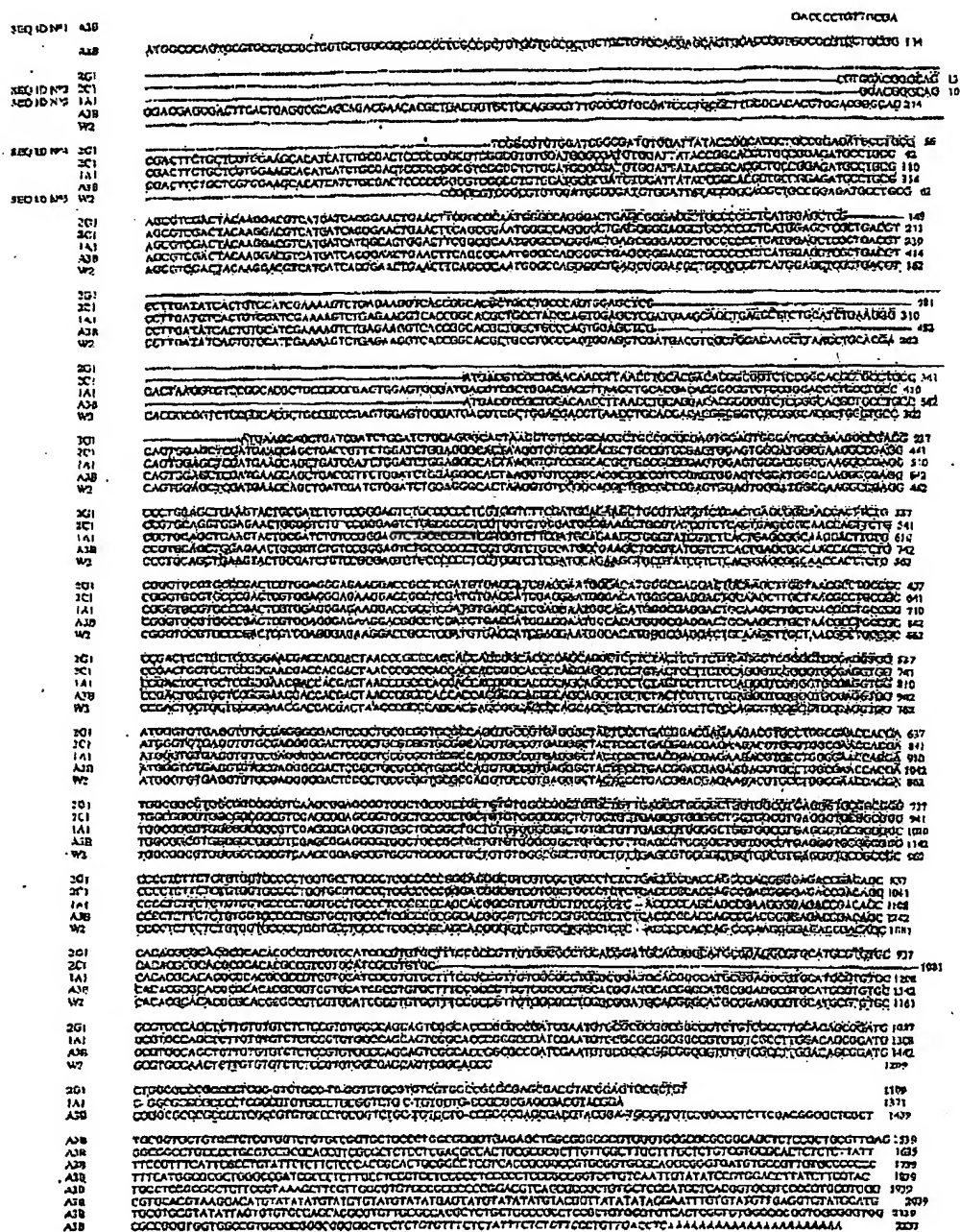


FIGURE 1

Alignment of the various cDNA sequences obtained

REPLACEMENT SHEET (RULE 26)

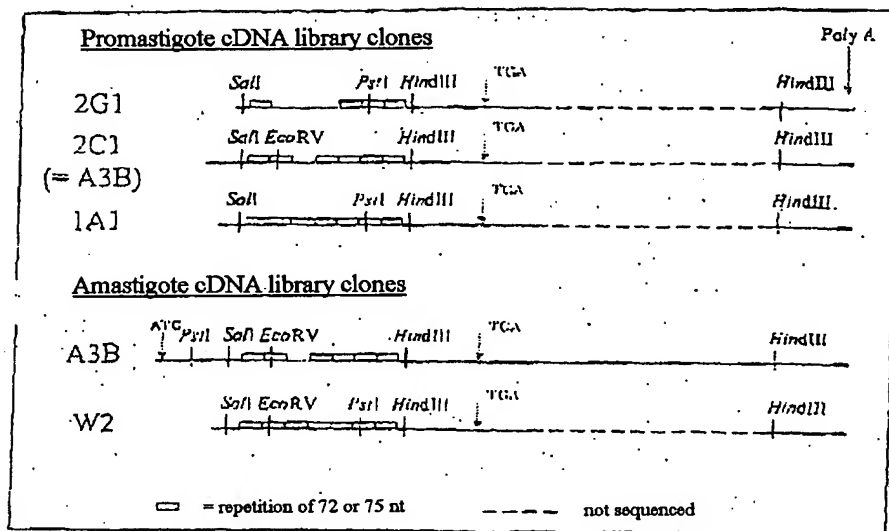


FIGURE 2

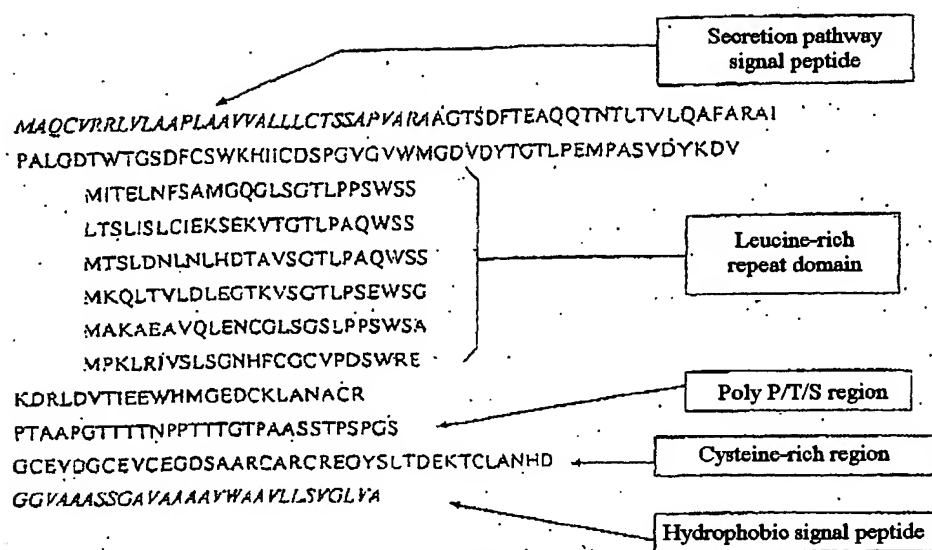


FIGURE 3A

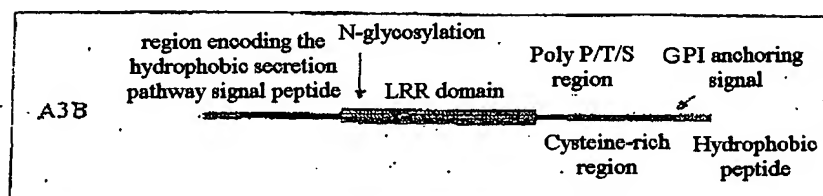


FIGURE 3B

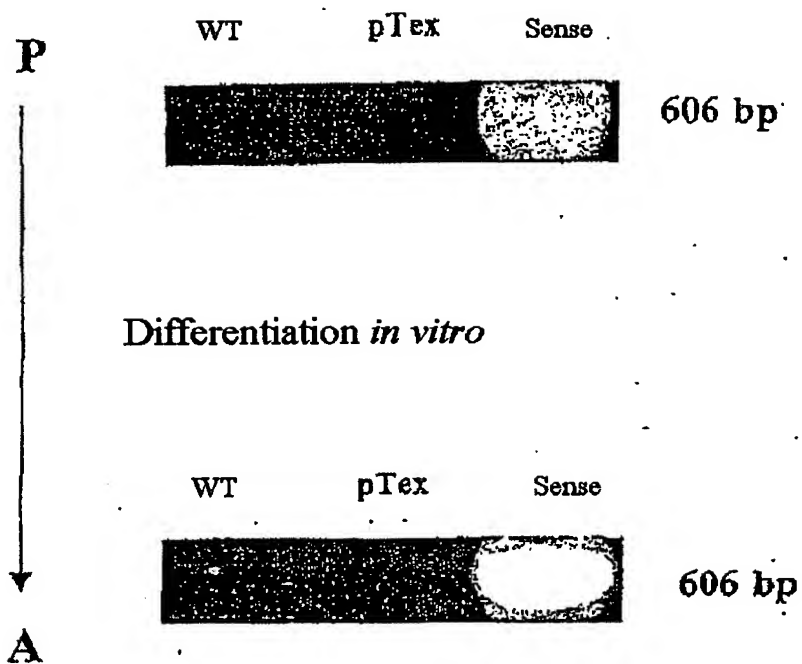


FIGURE 4

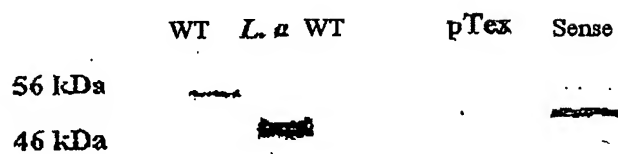
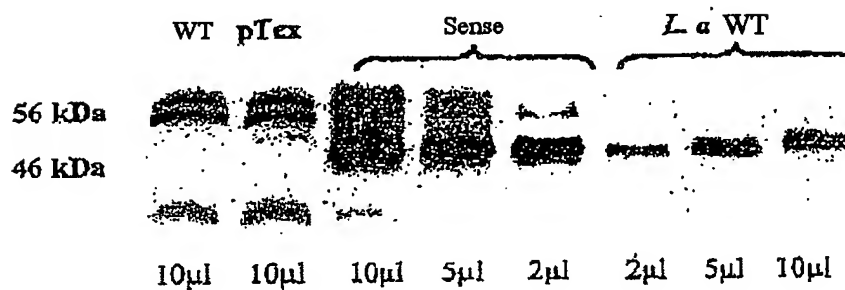
- Constitutive proteins- Excreted/secreted proteins (200 times concentrated culture supernatant)

FIGURE 5

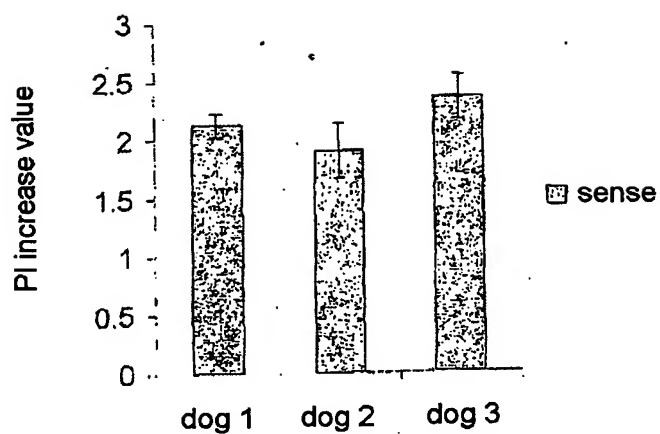


FIGURE 6A

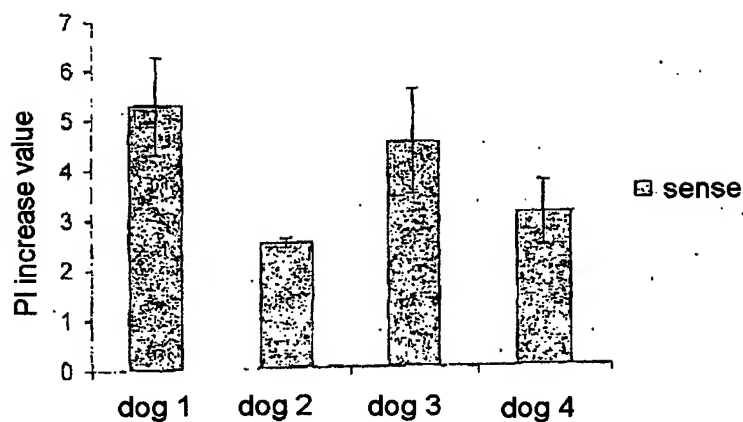


FIGURE 6B

PI = Parasitic Index. The variation in PI makes it possible to evaluate the infectious capacity of the Sense strain of interest, as a function of the percentage of infected macrophages and of the number of parasites per macrophage, relative to a control strain pTex.

SEQ ID No 1 (Nucleic sequence A3B)

[illegible]

SEQ ID No 2 (Nucleic sequence 2C1)

CGTGGACGGGCAGCGCACTTCTGCTCTGTGGAAAGCAATCATCTGCGACTCCCGGGCGCTCGGCGTGT
GATAGGGCGATATGTGGATTATAACCGGCACGCTGCGCGGASATGCTGGGAGCGCTCGCATATGAGGAG
GTACATGATACAGGAATTAAGTAACTCAAGCGCAATAGGGCGAGGGGCTAGCGGGGACGCTGCCCCCTC
ATGGAGCTCGCTGACGTCTTGATATCACTGTGCATCGAATAATCTGAGAAAGGTCAACGGGCACCGC
GGCTCGCCAGTGGAGCTCGATGACGTGCTGGAAGCAACCTTAACTCGACGACACGGCGGCTGTCCGG
CAAGCTGTGCTGCCAGTGGAGGTGCATGAGCAAGCACTGACCGTTCTGTGATCTGGAAGGCACTAAGGT
GTCCGGCACGCTGCGCTCGAGATGGAAATGGATGGCGAAGGCGGAGGCGGTGCAAGTGTGAGAACT
GGGCTGTGCTCGGGAGTCTGCCCGCTCGTGGTCTGCGATGCGGAAGCTCGGTATCGTCTCACTGA
CGGCGAACCACTTCTGCGGTGTGGGTGCCCGACTGTGCGAGGGAGAAGGACCGCGCTCGATGTGACG
ATCGAGGAATGGACATATGGCGGAGGACTGCAAGCTGTGCTAACAACGCTCGCGGCGCGACTGCTGTGCG
GGAACGACCAAGCACTAACCGCGCCACCAACCGCGACCCCAAGCGCTCTCTACTCTCTTCCAA
GGGTGCGGGTGTGAGAGTGGATGGGTGTGAGGCTGTGCGAGGGGGAATCGGCTGCGCGGTGCGCCAG
TGTGCTGTGAGGCTACTTCCCTGACGCGACGAGAAGACGTGCTGTGCGAACACGACATGGCGCGGTG
CGCGCGCTGTGAGGAGGAGCGGTGCTCGGCTGCTGTGTGCGGCGCTGTGCTGTGAGGCTGCGGCG
TGGTGGCTGTGAGGCTGCGCGGCGGCGCTCTCTCTGTGTGCTGCGCTGTGTGCTGTGCTGCGCGCG
GACGCGCGCTGTGCTGTGCGCTCTCTCAACCCCAACGACGCGAGCGGGAGACCGACAGCCACAGCGCG
ACCGCGACGCTGCGCTGTGCTGATGGGTGTGCG

Figure 7

SEQ ID No 3 (Nucleic sequence 1A1)

GGACGGGCGAGCGACTTCTGCTCGTGGAAAGCACATCATCTGCGACTCCCCGGCGTCCGGCGTGTGGA
TGGCGGATGTGGATTATACCGGCAACGCTGCGGAGATGCGTCCGAGCGTCACTACAAGGACGTC
ATGATCATGGCACTGGACTTCGGCGCAATGGCCAGGGACTGAGCGGACCGCTGCCCGCTCATGG
AGCTCCCTGACGCTCCTTGATGTCACTGTGGATGGAAGTCTGAGAAAGTCAACCGCACCGCTGCCT
ACCCAGTGGAGCTCGATGAAGCACTGACCTTCTGCACTGAAGGGCACTAAGGTGTCCGGCAGG
CTCCCGCCCGAGTGGAGTGGATGAGTCAAGGAGCTGATCGATCTGGATCTGGAGGGGCACTAA
GGACGCTGCTTCCCACTGGAGCTCGATGAAGGAGCTGATCGATCTGGATCTGGAGGGGCACTAA
GGTGTCCGGCAGCTGCGCGCCGAGTGGAGTGGGATGGGGAAGGCGGAGGCGCTGCAAGTGAAGT
ACTGCGATCTGTCCGGAGTCTGCCCGCTCGTGGTCTTCGATGCAAGAGCTGCGTATCGTCTCACT
GAGCGGCAACCACTTCTGCGGGTCCGTGCGGAGTCTGGAGGGGAGAAAGGACCGCGCTCGATGTGA
CCATCGAGGAATGGACATGGGCGAGGACTGCAAGCTTGTCTAAGCGCTGCCCGCGAGCTGCTGCTC
GGGGAAGGACCAAGGACTAAACCGGCCAACAACCGGCAAGCGGAGCGGCTCTCTCTCTCTCTCT
CAGGGTGGGGGTGCGAGGTGATGAGTGTGAGTGTGCGAGGGGGGACTCCGCTGCGCGGTGCGCG
AGGTGCGGTGAGGGCTACTCGCTGAGGAGCGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
GGCGCGCGCTGAGCGGAGCGGAGCGGTGGCTGGGCTGCTGTGTGGGCGCTGTGTGTGTGTGTGT
GGCTGT
CCAGCAGCGCGCTGCTGCTGCGCTCTCAACCGGCAAGCGGAGAGGGGAGAGAGAGAGAGAGAGAG
ACAGCGGCAAGCGCGCTGCTGCACTGCGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
GCGATGCGGAGGGCTGCACTGCGT
TCCGACCGCGCGCGCTGCTGCAATGT
GCGCGCGCTGCGCGCTGCGCGCTGCGCGCTGCGCGCTGCGCGCTGCGCGCTGCGCGCTGCGCGCT

SEQ ID No 4 (Nucleic sequence 2G1)

TCCGCTGT
ACAAGGACGTCATGATCAAGGAACTGAAGTTCGGGCAATGGGCGAGGGAAGTGAAGCGGAGCGCTG
GCCCGCTCATGAGGTCGATGAAGCAAGCTGATCGATGTGGATGTGGAGGGAAGTGAAGGTGTGTGT
ACCTGCGCGCGGAGT
GTCCGGGAGT
CCACTTCTGCGGGTGCCTGCGGAGTCTGTGGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
AATGGACATGGGCGAGGAGTGTGAAGCTTGTCTAAGCGCTGCGCGCGAGTGTGTGTGTGTGTGT
AECAGGACTAAGCGGCGCAACACCAAGGCGAGCGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
GGGTGCGAGT
TGAGGGCTACTCCCTGAGCGGAGGAG
CGTCAAGCGGAGCGGCTGT
CGTCAAGGAGT
CGT
CAGCAGCGCAGCGCAGCAGCGCTGT
ATGACAGGATGCGGAGCGCGCGGATCGAATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
CTAGCAGT
ATGT
GTGCGCTGT
TCCTGT

Figure 7 (continued)

SEQ ID No 5 (Nucleic sequence W2)

CCGCGCTCGGCGTGTGGATGGGCGATGTGGATTATACCGGGACGCTGCCGGAGAT
GCCTGCGAGCGTCGACTACAAGGACGTCATGATCACGGAACCTGAACCTTCAGCGC
AATGGGCCAGGGGCTGAGCGGGACGCTGCCCCCTCATGGAGCTCGCTGACGTCC
TTGATATCACTGTGCATCGAAAAGTCTGAGAAGGTCACCGGCACGCTGCCTGCC
AGTGGAGCTCGATGACGTCGCTGGACAACCTTAACCTGCACGACACGGCGGTCTC
CGGCACGCTGCCGCCGAGTGGAGTGGGATGACGTGCTGGACBACCTTAACCTG
CACGACACGGCGGTCTCCGGCAAGCTGCCTGCCAGTGGAGCTCGATGAAGCAG
CTGATCGATCTGGATCTGGAGGGCACTAAGGTGTCCGGCAGCTGCCGCCGAGT
GGAGTGGGATGGCGAAGGCCGAGGCCCTGCAGCTGAAGTACTGCCATCTGTGG
GGAGTCTGCCGCCCTCGTGTCTTCGATGCAGAAAGCTGCCGTATCGTCTCACTGAG
CGGCAACCACCTCTCGGGTGCCTGCCGAGTCTGTGGAGGGAGAAAGAACCGCT
CGATGTGACCATCGAGGAATGGCAGATGGGGGAGGACTGCAAGCTTGCTAACGC
CTGCCGCCCGAAGTGTCTCCGGGAACGACACGACTAACCGGCCACCACCACC
GGCACCCAGGAGCCTCCTCTACTCCTTCTCCAGGGTGGGGGTGCCAAGGTGG
ATGGGTGTGAGGTGTGCGAGGGGGACTCCGCTCCGCGGTGGCGCAAGGTCCGCT
AGGGCTACTCCTGACGGACGAGAAGACGTGCCCTGGCGAACCACGATGGCGGCGT
GGCGGCGGCGTCAAGCGGAGCGGTGGGTGCGGCTGCTGTGTGGGGGCTGTGT
GTTGATCGGTGGGCTGTGTGCGTGAAGGTGCCGCCGCCGCTCTTCTGTGTGT
CCCCTGGTGCCTGCCCTCGCCCCAGCACGGGTCTCTGCTGCCCTCTCACCCCCA
CCAGCCGAAGGGGAGACCGAACGACACGACAGGCACAGGGCCAGGGCGGTGTGCA
TCGCGTGTGCTTCCGCCGTTGTGCCCTGCGGGATGCCAAGGCATGCCGAGG
CGTGCATGGGTGTGGGGTCCCAACTCTGTGTGTCTCTCGTGTGGCCAGCAGTC
GGCACCC

Figure 7 (continued)

SEQ No 11 (Nucleic sequence IJ11 of Ldi)

GCCTGCTGCCGCTGGCGCTGTTGTGTGTGTGCTGGGGCCGCGCCACGCA
CACGCACGGTAGTGAGGGGGAGCCGCAGCGACCGACCGGGCGGAGCGGGC
GGGCGGAGGGGGGCGCTCCCGCCCGCTGGTCATGCTCTCTGTTTCGCTGG
CCGGCCTCTCTACGCCGCTGGCGTGGGCGGAGCTCCGCGCTGCSTATCGC
TCGCCCCCTCGCTGCCCTCCCTGCCCTCCCTCATCTGCACTGCTCCCTCC
CTCTCCCTCCCTCTACACTCCTCGCTGTCCCTCGGCGGACCTCCACGGA
CACGCAGACGTGCGTGCGCATACACACCACCCCTCACCTCGCTGCTGCTG
CTGTGACAGCTCTACGAGCCCTGCCAGTCTGCTGCGCCCCCGCCACCCGC
CTCTGTCCCCCGCACGAGGCTACCTACGACGTGCCGGCCACCCCGCTGTG
CCCGATAAGCTGAGCTGGCGCTCACGCCCGAGCAATCCCTCACGGATCT
GCTGCCGCGCCGCACTGCTCTTGACCCTGGCTGCGAATGGCGCTGTGCGT
GCGTCGGCTGGTGCTGGCGGCGACCTCGCCGCTGTGGTGGCGCTGCTGC
TGTGCACGAGCAGTGCGCCGGTGGCGCGTGTGCTGTGAAGGATGACTTC
ACTGCTGCGCAGCGGACGAACACGCTGGCGGTGCTGGAGGCGTTTGGGCG
TGCGATCCCTGAGCTTGGGAAGCTGTGGAAGGGCGACGACTTCTGC
TTTTGGGAGTCGCTCGTGTGCGATGTGACCGAAGTGTACTTGTGGGAA
ATCGGTGCGACGTATACCGGCACGCTGCCGGAGATGCCTGTGGACGTGCA
CTACACGGCCGTATGGTCAAGCACCTCGACTTTTCCCAAATGGGGCTGG
GGCTGAGCGGAACGCTGCCGGACAGCTGGAGCAGGCTGCAGGGACTGACC
TCACTTACGTTGTGCGGCTGCCGGCTGAGCGGTACGCTGCCCTCCCTCGTG
GCGCTCGATGAAGTCTTTGGTGTGCTTGTGGATTGAGAGTTGTGAAAGTG
TTACCGGCAAGCTGCCGCTGAGTGGAGCTCGATGAAATCGCTGAGAGAT
CTCCATCTGCATGGCGCGAAGGTTTCCGGCACGCTGCCGCTGAGTGGAG
CACGATGAAATCGCTGACCTTCTCGATCTGCAGGACACTCAGGTTACCG
GCAGTCTGCCGCTGAGTGGAGCTCAATGAAATCCATGACCATTCTCAGT
GACATGCGCGCGAAGGTTTCCGGCACGCTGCCACCCAGTGGAGCTCGAT
GACATGCTGAGCCITCTCAGTCTGGAGGGTACTCAGCTCTCCGGCAGC
TACCGCCCCAGTGGAGTGGATGACATCGCTGGTCAAGCTTTTCTCGCA
GGGTACTCAGGTCTCCGGCACTCTGCCGCGCAGTGGAGATCGATGTTGAA
TGCCGAGTTCTGCAGCTGGAGAACTGCGACCTGTCCGGCTGTTTGCCCCC
CGAGTGGGCTGCGATGCCGAAGCTGCGTCATGTGAACTTAAGGGCAACCA
GTTCCGCGGGTGTGTGCCGGAAGTGGTGGCTCAGAAGGCCGGTCTCGTTGT
GGAAATCGAGGATAAGCACACGGGCAACAGCTGCATTGCTGGTCCGGAAGT
CGCAACGACGACGACGACCACTGAACCCACGTCCACTGCGAGCCCAAC
AGCCACGCTTACCTCTGCCCGGAGACGGAGTGGAGGTGGATGGGTGTGA
GCTGTGCGATGGGGAAGTCCCGGGCAGGTTGCCAGGTGCCGTGAGGGCTA
CTTCTGACGAGGAGAGGAGCTGCTGGTGTACCGCGATGCCGGCGTTGT
GGCCGTGTGATCGGAGCGGCTGCTGCCGCTGTTGTGTGATGGCTGTGCT
GCTGAGCGTGGGGCTGGCGGCGTGGAGATGCCGCTGCTGTGCGCGCAGGC
GGCGGACCCCGCTGCGTGGCACACGACTGCGTGCTTGGCTGACGACCGG
CCCTGCAATTGGCGTGGCTGTGCGGCTGTGTGTGCAATGGCTGCTGACGGT
GCCTTTCGTCTGCTCTCGCTGCTCTGCTCTCTCCGCGTGTGAATGCT
GTGGGCTGTGTTTGGGGCTCTCGTSCGCGCTGCTGTACGGCTGCTGCTTC
TTCTCCACCTCTCTCTCGATGCCGCGAGGGAGGGGTGGCACGTGCGC
GTGTGCCGCTGCGCTTGGGAGTGCCTCTGTGTGTGGGCTTCACCACGTGC

Figure 7 (continued)

TACGGTCACGCCTTCTCGGCTGSCCACTCGCGGCGCTGAGGGCGGTGTSCC
CTTCCCCCTCGAGCGCCGTGCACTCTCTTCCGCGCGCCTGCGCGGGCTTCT
TCGTGCGCTGTGCTCAGCCGTGCGCTCTCACCTCTTTCCCTTTTCATTGCG
TTGTCTTCTCTCTTCTCCCCCGCACTGCGGTCTCCCCCTCCTGTGCGGTGC
GGTGGCGAGGCGGGTGACTTGCCGTGCGTCTCCCCCTTTCGTGGAGCGCT
GAGCCGATCCCCCTTCGGCCTCCCTCCTCCCTCCTCCCGTGGGTCTGTCT
GTTGTACATCGTGGACCGTCTCTTCGTGTTGCCTCTCCGCACCTTCCGCA
AATCTGCGCTCGCCTGTGCCGCCCTCTCGGACTTTATCCTTACTGTGATTGT
ATTCTCACGGTGCGTCTCCGTGTGTGTGTGTGCCACGCACCGCTTCTTCCA
TGTGTGTCCTTGCTTGCTCTCGTCTGCCCCCCCCCTCTGCCTCACACATT
CCGTGCGTGTGTGCATCACCGTTGGCGGGCGACATCGGTGCCCGTCCCTGC
CACCTCTACTCCCTCATTTCTCTTGCCACTTCGTGGGCGGTGCGTGCATGC
ATGGATGTATATACACGCATAGAGGGGTGGGGACGCGGGGGATCCTCTAGA
GTCGACCTGCAGGCATGCAAGCTTGCGTAATCATGGTCATAGCTGTTTCC
TGTGTGAAATTGTATCCGCTCACAATTCCACACAACATACGAGCCGGAAG
CATAAAGTGTAAGCCTGCGGTGCCCTAATGAGTGAGCTAACTCACATTAAT
TGGTTGCCCTC

Figure 7 (continued)

SEQ No 6 (Protein sequence A3B) (sense direction) 372 aa
 MAQCVRRLVLAAPLAHVALLCTSSAPVARAAGTSDFTAAQQTNTLTVLQAFARAI PAL
 GOTWTGSDFCSWKHII CDSPGVVWMDVDYTGTLPEMPASVDYKDVMITELNFSAMGGG
 LSGTLPPSWSSLTSLISLCIEKSEKVTGTLPAQWSSMTSLONLNLHDTAVSGTLEAQWSS
 MKQLTVLDLEGTKVSGTLPSEWSGMAKAEAVQLENCGLSGSLPPSWSAMPKLRIVSLSGN
 HFCGCVPD SWREKDRLOVTIEEWHMGEDCKLANACRPTAAPGTTTTNPPTTTGT PAASST
 PSPGSGCEVDGCEVCEGDSAARCARCREGYSLTDEKTCLANHDDGGVAAASSGAVRAAAVW
 AAVLLSVGLVA*

SEQ No 7 (Protein sequence 2C1) (sense direction) 287 aa
 MGDVDYTGTLPEMPASVDYKDVMITELNFSAMGQGLSGTLPPSWSSLTSLISLCIEKSEK
 VTGTLPAQWSSMTSLONLNLHDTAVSGTLPADWSSMKQLTVLDLEGTKVSGTLPSEWSGM
 AKAEAVQLENCGLSGSLPPSWSAMPKLRIVSLSGNHFCCGCVPD SWREKDRLOVTIEEWHM
 GEDCKLANACRPTAAPGTTTTNPPTTTGT PAASSTPSPGSGCEVDGCEVCEGDSAARCAR
 CREGYSLTDEKTCVANHDDGGVAAASSGAVAAAVWA VAVLLSVGLVA*

SEQ No 8 (Protein sequence 1A1) (sense direction) 311 aa
 MGDVDYTGTLPEMPASVDYKDVMINALDFGANGQGLSGTLPPSWSSLTSLMSLWIEKSEK
 VTGTLPTQWSSMKQLTLLHLKCTKVSGTLPPFWSGMTSLONLNLHDTAVSGTLPQWSSM
 KQLIDLDLEGTKVSGTLPPFWSGMAKAEALQLKYCDLGGSLPPSWSSMKLRIVSLSGNH
 FCGCVPD SWREKDRLOVTIEEWHMGEDCKLANACRPTAAPGTTTTNPPTTTGT PAASSTP
 SPGSGCEVDGCEVCEGDSAARCARCREGYSLTDEKTCLANHDDGGVAAASSGAVAAAVWA
 AVLLSVGLVA*

SEQ No 9 (Protein sequence 2G1) (sense direction) 238 aa
 MGDVDYTGTLPEMPASVDYKDVMITELNFGAMGQGLSGTLPPSWSSMKQLIDLDLEGTKV
 SGTLPPEWSGMAKAEALQLKYCDLGGSLPPSWSSMKLRIVSLSGNHFCGCVPD SWREKDR
 LOVTIEEWHMGEDCKLANACRPTAAPGTTTTNPPTTTGT PAASSTPSPGSGCEVDGCEV
 CEGDSAARCARCREGYSLTDEKTCLANHDDGGVAAASSGAVAAAVWA VAVLLSVGLVA*

SEQ No 10 (Protein sequence W2) (sense direction) 271 aa
 MGDVDYTGTLPEMFASVDYKDVMITELNFSAMGQGLSGTLPPSWSSLTSLISLCIEKSEK
 VTGTLPAQWSSMTSLONLNLHDTAVSGTLPPEWSGMTSLONLNLHDTAVSGTLPQWSSM
 KQLIDLDLEGTKVSGTLPPFWSGMAKAEALQLKYCDLGGSLPPSWSSMKLRIVSLSGNH
 FCGCVPD SWREKDRLOVTIEEWHMGEDCKLANACRPTAAPGTTTTNPPTTTGT PAASSTP
 SPGSGCEVDGCEVCEGDSAARCARCREGYS*

SEQ No 12 (Protein sequence IJ11 of Ldi) (sense direction) 464 aa
 MALCVRRLVLAATLAHVALLCTSSAPVARAAVKDDFTAAQRTNTLAVLEAFG
 RAIPELGKLWKGDDFCFWBSVVVRCDRSVLGGKSVRRIPARCRRCLWTSTTRPSW
 SSTSTFFKWGWGWAERCRTAGAGCRDWPHLRCRAAAWAYRCPPRGARWSLWCR
 CGLRVVKVLPASCRLSGARWNRWEISICMARRFPARCRLSGARWNRWPPSICRTL
 RLPVAVCRLSGAQWNPWPFSVWMARRFPARCHPSGARWHRWAFSVWRVLSPPAR
 YRPSGSGMTSLVTLFLQGTQVSGTLPQWRSMLNAEFLQLENCGLSGCLPPEWAA
 MPKLRHVELKGNQFAGCVPD SWAQKAGLVVEIEDKHTGNSCIA GADCATTTTTTT
 EPTSTASPTATPTSA PETECEVDGCEVCDGDSAARCARCREGYFLDERTCLVYRD
 GGVA VASIGAAAAAVVCMVAVLLSVGLAA*

Figure 7 (continued)

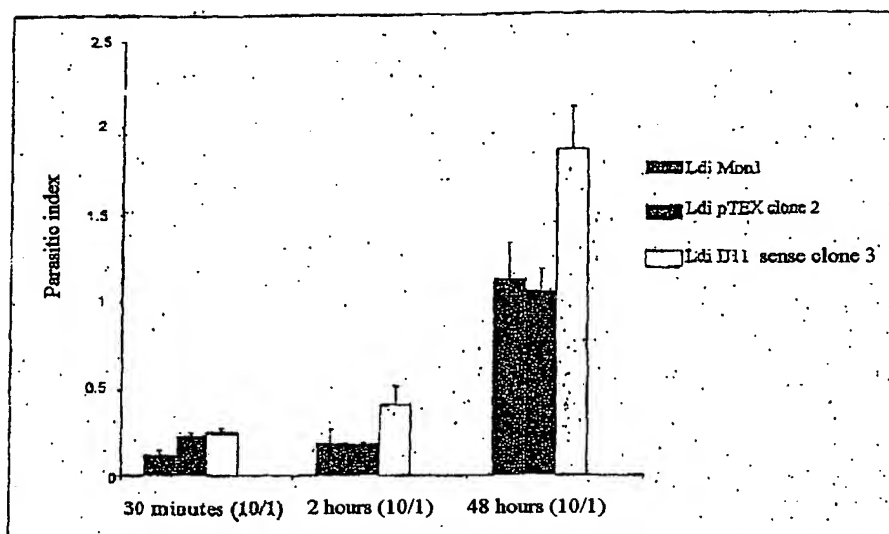


Figure 8